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## Growth Factor Requirements of Saccharolytic Butyl Alcohol-Acetone Bacteria

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## Growth Factor Requirements of Saccharolytic Butyl Alcohol-Acetone Bacteria

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### INTRODUCTION

In a Letter to the Editors (1) we reported the growth factor requirements of three saccharolytic butyl alcohol-acetone bacteria. Further studies on these same organisms have now been completed and are considered in this paper.

The nutritive requirements of the acetone-butyl alcohol bacteria and the factors involved in the production of solvents from synthetic or semi-synthetic media have been widely investigated (2-10).

In the present work the three cultures studied have been designated B<sub>1</sub>, B<sub>2</sub>, and BA<sub>1</sub>, pending their actual classification. Culture B<sub>1</sub> was an old stock culture, whereas cultures B<sub>2</sub> and BA<sub>1</sub> were recently isolated from soil. All three organisms attacked molasses readily but fermented corn starch slowly. B<sub>1</sub> and B<sub>2</sub> required only biotin for maximum growth and BA<sub>1</sub> needed both biotin and *p*-aminobenzoic acid. On the synthetic glucose-biotin medium, B<sub>1</sub> produced solvents in amounts which compared favorably with those from the molasses fermentation; this was considered normal. Cultures of B<sub>2</sub>, however, gave a subnormal yield of solvents on the same medium. The BA<sub>1</sub> organism produced almost normal quantities of acetone and butyl alcohol from glucose when both biotin and *p*-aminobenzoic acid were present.

### EXPERIMENTAL

*Cultures and Inoculum.* The stock cultures were maintained in sterile soil. Tubes of freshly boiled sterile potato mash were inoculated while hot with a loopful of the soil culture, allowed to stand for one minute, cooled, and incubated at 37°C. After 24 hours, a transfer was made from each tube into a medium containing 2.55% glucose and 0.45% high test cane molasses. This ratio was chosen because preliminary

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experiments had shown that growth did not occur when the molasses provided less than 10% of the total sugar. The glucose-molasses culture was centrifuged after 24 hours incubation, the cells suspended in sterile distilled water, recentrifuged, and resuspended in sterile water. One-tenth ml. of this preparation was used to inoculate 10 ml. of medium in the growth requirements tests. For the fermentations, 3.0 ml. of the 24-hour glucose-molasses cultures were employed for inoculating 150-ml. portions of molasses, glucose-molasses, or synthetic medium.

*Basal Medium.* This was composed of 3.0% glucose, 0.3% ammonium sulfate, Speakman's salts\* (1.0 ml. per 10 ml. of medium), and 0.18% calcium carbonate. Biotin as the hydrolyzed methyl ester, and *p*-aminobenzoic acid were added in quantities of 0.001  $\mu$ g. and 0.2  $\mu$ g. per ml. of medium, respectively.

*Incubation and Measurement of Growth.* The cultures under investigation for growth requirements were placed in a jar containing 2–3 inches of moistened oats evacuated to provide anaerobic conditions, and incubated at 37°C. The amount of sugar consumed during the period of incubation was used as an index of growth. Turbidity measurements were tried but the excess of  $\text{CaCO}_3$  in suspension interfered with this procedure. The fermentations were carried out in 250-ml. Erlenmeyer flasks, and were freed of oxygen by bubbling nitrogen through them for 10–15 minutes. They were then sealed from the air, and allowed to incubate at 37°C.

*Methods of Analysis.* Sugar was determined by the Shaffer-Somogyi method (11). The iodoform reaction was used for the determination of acetone and carried out as follows: 25 ml. of 1 *N* NaOH and 25 ml. of 0.1 *N*  $\text{I}_2$  were added to a sample containing not more than 0.35 millimoles of acetone and the temperature of the mixture was kept at 0°C. for ten minutes. The reaction was carried out in glass-stoppered bottles. The solution was acidified with an excess of 0.2 ml. of 2 *N*  $\text{H}_2\text{SO}_4$ . The excess iodine was titrated with 0.05 *N*  $\text{Na}_2\text{S}_2\text{O}_3$  using starch as an indicator. A blank on the reagents was run simultaneously. The acetone was calculated as follows:

$$\frac{(\text{Titration of blank} - \text{Titration of sample}) \times \text{Normality of } \text{Na}_2\text{S}_2\text{O}_3}{6} = mM \text{ Acetone}$$

The butyl and ethyl alcohols and the volatile acids were estimated by the partition method of Osburn, Wood, and Werkman (12). The alcohol distillate was first oxidized with potassium dichromate and sulfuric acid and steam distilled.

Carbon dioxide and hydrogen, lactic acid and 2,3-butylene glycol were determined according to the methods used by Mickelson and Werkman (13).

*Cultural and Morphological Characteristics.* The three organisms are obligate anaerobes and show very slight differences in morphology. They all form spores with enlargement of the cell, either terminal or subterminal. Surface colonies on glucose agar plates are circular or irregular, raised and moist.

Among the biochemical reactions studied were nitrogen fixation, hydrogen sulfide production, hydrolysis of certain proteins, nitrate reduction, and fermentation of a selected group of carbohydrates and related compounds.

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\* Speakman's salts: 0.25%  $\text{K}_2\text{HPO}_4$ , 0.25%  $\text{KH}_2\text{PO}_4$ , 0.10%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005%  $\text{NaCl}$ , 0.005%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.005%  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ .



Table I gives the results of the cultural studies.

TABLE I  
*Biochemical Reactions of Cultures Designated as B<sub>1</sub>, B<sub>2</sub>, and BA<sub>1</sub>*

|                 | Dextrose | Glycerol | Xylose | Arabinose | Rhamnose | Mannitol | Corn starch | Pectin | Casein hydrolysis | Albumin hydrolysis | Gelatin liquefaction | Nitrate reduction | Hydrogen sulfide production | Nitrogen fixation |
|-----------------|----------|----------|--------|-----------|----------|----------|-------------|--------|-------------------|--------------------|----------------------|-------------------|-----------------------------|-------------------|
| Control         | —        | —        | —      | —         | —        | —        | —           | —      | —                 | —                  | —                    | —                 | —                           | —                 |
| B <sub>1</sub>  | AG       | —        | AG     | AG        | AG       | AG       | ±           | —      | —                 | —                  | —                    | —                 | —                           | —                 |
| B <sub>2</sub>  | AG       | —        | AG     | AG        | AG       | AG       | ±           | —      | —                 | —                  | —                    | —                 | —                           | —                 |
| BA <sub>1</sub> | AG       | —        | AG     | AG        | —        | —        | ±           | —      | —                 | —                  | —                    | —                 | —                           | —                 |

AG — acid and gas.

± — little acid and gas.

— — no acid or gas.

*Nutrient Requirements.* Thiamine, riboflavin, pyridoxin, biotin, pantothenic acid, nicotinamide, inositol, and *p*-aminobenzoic acid were tested for their growth stimulating effect. A medium containing all of these growth factors in addition to glucose and salts supported growth of all three organisms. By eliminating one vitamin at a time from this medium, it was observed that only biotin was essential for strains B<sub>1</sub> and B<sub>2</sub>, while BA<sub>1</sub> required both biotin and *p*-aminobenzoic acid. To confirm these findings the organisms were grown in combinations of the basal medium with each single vitamin. B<sub>1</sub> and B<sub>2</sub> were able to consume the sugar in the glucose-biotin mixture. BA<sub>1</sub> showed no growth in the medium containing just biotin or *p*-aminobenzoic acid, but utilized most of the sugar in the presence of both factors.

Table II gives the residual sugar in some of the media tested at the end of seven days. The figures are self-explanatory.

The stimulating action of biotin on the growth of these organisms was found to be inhibited or entirely suppressed by the addition of a small amount of egg white to the medium.

*Fermentations.* Fermentations were carried out in volumes of 150 ml. of media. The yields of solvents were determined as follows: An aliquot portion of the fermentation liquor was acidified with sulfuric acid before distillation using Congo red as indicator. This precaution was necessary because some sugar might be present. The

TABLE II

*Effect of Biotin and p-Aminobenzoic Acid (= PABA) on the Growth of Cultures Designated as B<sub>1</sub>, B<sub>2</sub>, and BA<sub>1</sub>*

Residual sugar, in percentage, after 7 days

| Culture         | B.M.* only | B.M. + Biotin | B.M. + PABA | B.M. + Biotin<br>+ PABA |
|-----------------|------------|---------------|-------------|-------------------------|
| B <sub>1</sub>  | 2.85       | 0             | 3.13        | 0.2                     |
| B <sub>2</sub>  | 2.87       | 0             | 2.96        | 0.2                     |
| BA <sub>1</sub> | 2.91       | 2.89          | 2.96        | 0.86                    |

\* Basal medium, control.

distillate which was collected in a small amount of ice-cold water, contained the volatile acids and neutral volatile products. It was subsequently neutralized with sodium hydroxide, using phenolphthalein (dry powder added) as indicator, and re-distilled. The second distillate, which contained acetone and butyl and ethyl alcohols, was collected under water in a volumetric flask which was kept in an ice bath. Aliquot portions of this neutral distillate were used for the determination of acetone and butyl and ethyl alcohols.

The residues from the two distillations were combined, sulfuric acid added to the acid end point of Congo red, and steam-distilled. The distillate contained the volatile acids, butyric and acetic. These substances were then determined quantitatively by the partition method.

When a complete analysis of the products of fermentation was desired, the technique outlined in (12) was followed. The residual carbon dioxide was first removed and weighed before the initial distillation. Lactic acid and 2,3-butylene glycol were determined on an aliquot portion of the fermentation liquor. Lactic acid was determined on the residue of the 2,3-butylene glycol distillation.

The results obtained when B<sub>1</sub>, B<sub>2</sub>, and BA<sub>1</sub> were cultured on cane molasses and synthetic media are shown in Table III.

TABLE III

*The Yield of Solvents Produced by Strains of B<sub>1</sub>, B<sub>2</sub>, and BA<sub>1</sub>, when Cultured on High-Test Molasses and Synthetic Media*

| Culture         | Medium              | Products in mM. per 100 mM. glucose fermented |               |               |              |             |
|-----------------|---------------------|-----------------------------------------------|---------------|---------------|--------------|-------------|
|                 |                     | Acetone                                       | Butyl alcohol | Ethyl alcohol | Butyric acid | Acetic acid |
| B <sub>1</sub>  | Molasses, H.T.      | 24.50                                         | 54.00         | 13.40         | 1.80         | 6.42        |
| B <sub>1</sub>  | Glucose-biotin      | 17.80                                         | 44.80         | 4.88          | 2.10         | 7.48        |
| B <sub>2</sub>  | Molasses, H.T.      | 18.60                                         | 58.82         | 11.20         | 7.14         | 7.99        |
| B <sub>2</sub>  | Glucose-biotin      | 4.47                                          | 28.77         | 0.00          | 25.41        | 26.46       |
| BA <sub>1</sub> | Molasses, H.T.      | 17.55                                         | 46.90         | 9.90          | 8.19         | 7.56        |
| BA <sub>1</sub> | Glucose-biotin-PABA | 7.80                                          | 53.40         | 2.74          | 9.90         | 10.34       |

One of the strains, B<sub>1</sub>, was cultured serially at 48-hour intervals for 150 generations on the basal medium containing glucose and biotin. Table IV gives complete analyses of fermentations of molasses and

TABLE IV

*A Complete Fermentation Balance for the 78th Generation of Strain B<sub>1</sub> in Synthetic Medium when Cultured on High-Test Molasses and Glucose-Biotin Media*

| Culture        | Medium         | Products in <i>mM.</i> per 100 <i>mM.</i> glucose fermented |                |         |               |               |             |              |                     |             | Carbon recovery | O/R Index |
|----------------|----------------|-------------------------------------------------------------|----------------|---------|---------------|---------------|-------------|--------------|---------------------|-------------|-----------------|-----------|
|                |                | CO <sub>2</sub>                                             | H <sub>2</sub> | Acetone | Butyl alcohol | Ethyl alcohol | Acetic acid | Butyric acid | 2,3-Butylene glycol | Lactic acid |                 |           |
| B <sub>1</sub> | Molasses, H.T. | 193.00                                                      | 95.00          | 19.60   | 56.30         | 6.22          | 5.50        | 4.80         | 7.65                | —           | 92.0            | 0.96      |
| B <sub>1</sub> | Glucose-biotin | 204.00                                                      | 138.20         | 19.00   | 45.20         | 10.10         | 23.70       | 6.50         | 6.04                | 1.08        | 94.0            | 0.99      |

glucose-biotin media by the B<sub>1</sub> culture. The 78th transfer in glucose-biotin medium was used as inoculum in these fermentations. A fermentation study was then made by inoculating a flask containing glucose-biotin medium with the 150th generation of culture B<sub>1</sub>, to

TABLE V

*Yield of Solvents Produced by 150th Generation of Strain B<sub>1</sub> in Synthetic Medium when Cultured in Glucose and Biotin*

| Culture        | Medium         | Products in mM. per 100 mM. of fermented glucose |               |               |             |              |
|----------------|----------------|--------------------------------------------------|---------------|---------------|-------------|--------------|
|                |                | Acetone                                          | Butyl alcohol | Ethyl alcohol | Acetic acid | Butyric acid |
| B <sub>1</sub> | Glucose-biotin | 1.00                                             | 21.45         | 17.90         | 32.90       | 47.20        |

observe whether or not the long period of growth on the glucose-biotin medium had altered the fermentation capacity of this strain. The data are given in Table V. The results show a subnormal yield of solvents and a correspondingly higher acidity. The amount of ethyl alcohol is also greater than usual.



## DISCUSSION

Many studies have been made on the growth factor requirements of the butyl alcohol-acetone bacteria of the *Cl. acetobutylicum* type. In most instances biotin has been found essential for these organisms, but in no case has it been the only growth factor required.

The three strains under consideration in this paper are closely related as to morphological and biochemical characteristics. On the basis of their ability to ferment starch they are not identical with a strain of *Cl. acetobutylicum* in this laboratory. The three organisms are highly saccharolytic, non-proteolytic, and non-nitrogen fixing anaerobes. They all attack dextrose, arabinose, and xylose, but only two of them ferment rhamnose and mannitol. None of them can decompose pectin or glycerol.

All three organisms required biotin for optimum growth. Only one, the BA<sub>1</sub> strain, needed *p*-aminobenzoic acid in addition to biotin.

A comparison was made between yields of solvents in synthetic media and in a medium composed of high test cane molasses which is considered best for maximum solvent yields. Table III shows that the yield of acetone was generally lower in the synthetic medium. The difference is especially pronounced in the case of the B<sub>2</sub> strain which also gave a poorer yield of butyl alcohol and a higher acidity in the glucose-biotin medium. The presence of other B-vitamins in the medium had no effect on the production of solvents.

The B<sub>1</sub> organism was carried in the synthetic medium for as many as 150 transfers and maximum growth was maintained. However, a fermentation by the 150th generation on glucose-biotin medium gave unaccountably high acidity and less acetone and butyl alcohol. The culture was pure insofar as could be determined from subsequent platings. This finding suggests the possibility that the organism might have lost its ability to produce solvents after a rather long series of repeated transfers in synthetic medium.

## SUMMARY

Three strains of saccharolytic butyl alcohol-acetone bacteria have been studied for their growth requirements and the effect of the composition of the medium on the yield of solvents. Two of these strains required only biotin for maximum growth and the third needed both biotin and *p*-aminobenzoic acid. Thiamine, riboflavin, pyridoxin, pantothenic acid, nicotinamide, and inositol were without effect.



Fermentations by these bacteria in high test cane molasses and synthetic media gave approximately the same results. Acetone production was slightly lower in the synthetic media.

One strain, B<sub>1</sub>, was carried through 78 transfers at 48-hour intervals on the synthetic medium and still gave good solvent yields. However, after 150 transfers it no longer gave good solvent yields.

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